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The majority of lactococcal plasmids carry a highly related replicon

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DNA sequence analysis and Southern hybridizations, together with complementation experiments, were used to study relationships between lactococcal plasmid replicons. pWVO2, pWVO4 and pWVO5, which co-exist in *Lactococcus lactis* subsp. *cremoris* Wg2, and pIL7 (isolated from another strain) all contained a functional replication region which appeared to be very similar to that of some known lactococcal plasmids. They contain a gene encoding a highly conserved RepB protein (60–80% amino acid identity between pWVO2, pWVO4 and pWVO5), which is essential for replication. When supplied *in trans*, *repB* of pWVO2 complemented a *repB* deficiency of pWVO5. Upstream of the *repB* gene, all these plasmids contain a strongly conserved region including a 22 bp sequence tandemly repeated three-and-a-half times, and an A/T-rich region. The similarity with pWVO2, which is known to replicate via a theta mechanism, suggests that all plasmids of this family are capable of theta replication. Southern hybridizations revealed that many lactococcal strains contain plasmids of this family.

Keywords: lactococcal plasmids, plasmid replication, plasmid incompatibility

INTRODUCTION

Lactococcal strains generally carry a number of different plasmids, varying in size from approximately 2 kb to over 100 kb, some of which specify traits of major interest for the dairy industry. A consequence of the plasmid location is that the desired trait is lost when the plasmid is lost from the population. This is a problem of both fundamental and applied interest. Plasmid loss is a frequently observed problem in Gram-positive bacteria, such as *Bacillus subtilis* (Bron *et al.*, 1991; Gruss & Ehrlich, 1989) and *Staphylococcus aureus* (Novick, 1989), and has also been observed in lactococci (Otto *et al.*, 1983). Recently, we showed that the cloning of large DNA fragments in rolling-circle (RC) plasmids results in poor maintenance in *Lactococcus lactis* (Kiewiet *et al.*, 1993). When the streptococcal theta plasmid pAMβ1 was used for similar experiments, the resulting recombinant plasmids were, however, stably maintained. Based on this observation, we anticipated that endogenous theta plasmids from lactococci would be valuable for the development of stable (food-grade) cloning vectors in lactic acid bacteria.

One of the possible causes of plasmid loss may be the complex mixture of plasmids in most lactococcal strains. It is conceivable that in strains harbouring related plasmids, interaction at the level of replication or partitioning would result in incompatibility. For future development of stable food-grade plasmid vectors it is, therefore, important to analyse the relationships and interactions between plasmids within one strain. In the present work we analysed the replication regions of a number of lactococcal plasmids. Specifically, we addressed the question whether the various plasmids which co-exist in *L. lactis* subsp. *cremoris* Wg2 are related.

A number of lactococcal plasmids, other than those of the RC-type, have recently been analysed. These plasmids, which show a high degree of similarity, comprise the 8·7 kb cryptic plasmid pCI305 (Hayes *et al.*, 1991), the lactose plasmid pSK11L (Jahns *et al.*, 1991), the citrate plasmid pSL2 (Horng *et al.*, 1991), the 3·8 kb cryptic plasmid pWVO2 (Kiewiet *et al.*, 1993), a 28 kb cryptic plasmid that has been used to construct the nisin-resistant plasmid pVS40 (Von Wright *et al.*, 1990; GenBank accession number L02920), and the bacteriophage-resistance plasmid pCI528 (GenBank accession number L06274). One of these, the cryptic plasmid pWVO2, was derived from *L. lactis* subsp. *cremoris* strain Wg2, which carries a total of five different plasmids (Otto *et al.*, 1983).

Abbreviation: RC, rolling circle.

The EMBL accession numbers for the nucleotide sequences reported in this paper are Z25475 (pIL7), Z25476 (pWVO4) and Z25477 (pWVO5).

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The smallest of these, the RC-type plasmid pWVO1 (2·17 kb), has been studied extensively, and a large set of multipurpose cloning vectors is based on it (Leenhouts *et al.*, 1991; Kok, 1991). Of the other plasmids from this strain, denoted pWVO2 (3·8 kb), pWVO3 (7 kb), pWVO4 (19 kb) and pWVO5 (27 kb), only the first has been studied in some detail. Recently, we showed that pWVO2 replicates via a theta mechanism (Kiewiet *et al.*, 1993). Hitherto, no sequence data on the replication regions of pWVO3, pWVO4 and pWVO5 have been available. pWVO5 is of particular interest, since it carries the gene specifying the proteinase required for casein breakdown in cheese production (Otto *et al.*, 1983; Kok *et al.*, 1988). Loss of proteinase activity is frequently observed during cheese production and appears to result from the loss of a plasmid specifying the proteinase gene (Otto *et al.*, 1983).

In this paper we describe the isolation and characterization of functional replication regions of pWVO4, pWVO5 and pIL7. The latter plasmid, extracted from *L. lactis* IL594, specifies a restriction/modification system that renders carrier strains resistant to a number of bacteriophages (Chopin *et al.*, 1984). We were unable to clone the replication functions of pWVO3, and this plasmid was therefore not included in these studies. Since this type of plasmid is closely related to the theta-replicating plasmid pWVO2 (Kiewiet *et al.*, 1993), this family of plasmids is likely to be valuable for the development of stable, food-grade cloning vectors, and for studies on the mechanisms underlying plasmid instability and (in)compatibility in lactococci.

METHODS

Bacterial strains, growth conditions and plasmids. All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were transformed as described by Mandel & Higa (1970) and plated on LB agar or grown on LB medium (Sambrook *et al.*, 1989), supplemented with 100 μg erythromycin ml^{-1} or 75 μg ampicillin ml^{-1} when required. Tests for replication proficiency of plasmid derivatives were carried out in *L. lactis* subsp. *lactis* MG1363 (Gasson, 1983). Cells, grown on M17 medium (Terzaghi & Sandine, 1975), were transformed by electroporation (Holo & Nes, 1989) and plated on M17 agar, containing 0·5 M sucrose and 5 μg erythromycin ml^{-1}.

Isolation of plasmid DNA; DNA manipulations. Isolation of plasmid DNA by the alkaline lysis method was essentially as described by Sambrook *et al.* (1989) with some minor modifications. Complete lysis of cells was achieved by resuspending cell pellets in solution I supplemented with lysozyme in a minimal volume of 7·5 % of the original volume. This step was followed by an incubation at 55 °C for 10–15 min. For large-scale plasmid isolations, DNA was precipitated with 2-propanol prior to phenol/chloroform extraction in order to reduce the volume. Plasmids were isolated from 1 % (w/v) agarose gels using the Prep-a-gene kit as recommended by the manufacturer (BioRad). DNA sequencing was performed by the dideoxynucleotide chain-termination method of Sanger *et al.* (1977). All sequences were determined on both strands. PCR reactions were carried out as described by Sambrook *et al.* (1989). Deletions for determining the region required for replication of pJR7 were made with Exo III and mung bean nuclease as described by the supplier (Stratagene).

Oligonucleotides. To sequence the replication regions of pWVO4, pWVO5 and pIL7 a set of ten oligonucleotides was designed. The sequence of these was based on homologous regions between the nucleotide sequences of the *rep* genes of pWVO2, pCI305, pSL2 and pSK11L. Oligonucleotides were constructed in such a way that at locations where different nucleotides were present between the different sequences, these different nucleotides could be incorporated (indicated in parentheses in Table 2), resulting in a mixture of different oligonucleotides in equal amounts. Oligonucleotides P1 through P5 were designed to prime DNA synthesis in one direction; the partly complementary oligonucleotides P6 through P10 were designed to prime DNA synthesis in the other direction. These sets of oligonucleotides enabled us to obtain the sequences for both strands. The position of these primers, relative to the amino acid sequences encoded by the *rep* genes, is indicated in Fig. 1.

Regions outside the *rep* gene were sequenced with oligonucleotides based on the sequences obtained from oligonucleotides P1 through P10 and completed for both strands.

Southern hybridizations. Southern transfers were carried out as described by Sambrook *et al.* (1989) using Gene Screen Plus membranes as carrier (NEN-Research Laboratories). DNA fragments used as probes were prepared by PCR of complementary DNA using primers 1 and 10 (Table 2) and pWVO2 as a template. Probe labelling, hybridization and the detection of complementary DNA were conducted using the ECL gene detection system (Amersham) as recommended by the manufacturer. Hybridization conditions were as described for homologous probes (42 °C, 0·5 M NaCl).

RESULTS

Cloning of replication regions of plasmids pWVO4, pWVO5 and pIL7

Plasmids pWVO4 and pWVO5 were isolated from agarose gels on which the complex plasmid mixture of *L. lactis* subsp. *cremoris* Wg2 was separated. pIL7, originally present in *L. lactis* IL594, was isolated from a lactococcal strain carrying only this plasmid (Chopin *et al.*, 1984). Different approaches were used for the cloning of the replication regions of the various plasmids that were compared in this study.

pWVO4/pJRO4. Plasmid pWVO4 was digested with *Bgl*II. This resulted in three large fragments which were cloned into the *Bgl*II site of pMTL23E (Table 1). Since pMTL23E cannot replicate in *L. lactis*, this vector was used to screen for DNA fragments that enabled replication in this bacterium. Em^R transformants were initially selected in *E. coli* JM101. Only recombinant plasmids which carried the 4·5 kb *Bgl*II fragment from pWVO4, in either orientation, were able to support replication after subsequent transfer to *L. lactis*. One of these plasmids, pJRO4 (Table 1), was used for further analysis.

pWVO5/pJRO5. Plasmid pWVO5 was digested with *Eco*RI. The resulting seven fragments were inserted into the *Eco*RI site of pMTL23E. The ligation mixture was used to transform *L. lactis* directly. Transformants

Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Properties	Source or reference
E. coli		
JM101	*sup*E *thi*Δ(*lac-pro*AB) [F′ *tra*D36 *pro*AB *lacI*^{q} *lacZ*ΔM15	Yanisch-Perron *et al.* (1985)
L. lactis		
MG1363 subsp. *lactis*	Plasmid-free, Lac$^-$ Prt$^-$	Lab. collection
subsp. *cremoris* Wg2	Industrial strain, containing pWVO1–pWVO5	Lab. collection
subsp. *cremoris* E8L	Natural strain, cryptic plasmid complement	Visser *et al.* (1986)
subsp. *cremoris* H61	Natural strain, cryptic plasmid complement	Okamoto *et al.* (1985)
subsp. *cremoris* F16	Natural strain, cryptic plasmid complement	Okamoto *et al.* (1983)
subsp. *cremoris* Hpl	Natural strain, cryptic plasmid complement	Exterkate (1975)
subsp. *cremoris* 916	Natural strain, cryptic plasmid complement	Lab. collection
subsp. *cremoris* Cla2	Natural strain, cryptic plasmid complement	Lab. collection
subsp. *cremoris* 109	Natural strain, cryptic plasmid complement	Lab. collection
L10	Natural strain, cryptic plasmid complement	Lab. collection
P8-2-47	Natural strain, cryptic plasmid complement	Lab. collection
9B4	Natural strain, cryptic plasmid complement	Lab. collection
Plasmids		
pWVO2	3·8 kb, cryptic, from *L. lactis* Wg2	Otto *et al.* (1983)
pWVO4	6·5 kb, cryptic, from *L. lactis* Wg2	Otto *et al.* (1983)
pWVO5	27 kb, specifies proteinase, from *L. lactis* Wg2	Otto *et al.* (1983)
pIL7	31 kb, specifies restriction/modification system, from *L. lactis* IL594	Chopin *et al.* (1984)
pMTL23E	Ap^R Em^R, 4·0 kb, pMTL23 derivative containing the Em^R gene in the *Cla*I site (pE194*cop*6 coordinates 3140–1939) and the T1T2 transcriptional terminator (550 bp) in the *Eco*RI site; not able to replicate in *L. lactis*	Kiewiet *et al.* (1993)
pJRO4	pMTL23E, carrying a 4·5 kb *Bgl*II fragment of pWVO4, capable of autonomous replication in *L. lactis*	This work
pJRO5	pBSKII$^-$, carrying a 7 kb *Eco*RI fragment of pWVO5, capable of autonomous replication in *L. lactis*	This work
pJRO55	pMTL23E, carrying a 3·9 kb *Hin*dIII fragment of pWVO5, not capable of autonomous replication in *L. lactis*	This work
pJR7	pMTL23E, carrying a 3·2 kb *Sau*3A fragment of pIL7, capable of autonomous replication in *L. lactis*	This work

selected on erythromycin-containing plates were analysed for their plasmid content. All appeared to contain the same 7 kb *Eco*RI insert, which was found in both orientations. Plasmids containing this insert in one orientation, pJRO5 (Table 1) were used for further analysis.

pIL7/pJR7. In an experiment aimed at the cloning of fragments from large (theta-replicating) plasmids that would increase segregational plasmid stability, a 3·2 kb *Sau*3A fragment of pIL7 was isolated from a partial *Sau*3A digest. This fragment was subsequently cloned into the *Bam*HI site of pMTL23E. Plasmid DNA containing this insert was isolated from *E. coli* and used to transform *L. lactis*. Plasmid-containing transformants were obtained, indicating that the 3·2 kb insert carried a region of pIL7 that was capable of sustaining autonomous replication in *L. lactis*. This plasmid, pJR7 (Table 1), was used for further analysis.

DNA sequences of the replication regions of pJRO4, pJRO5 and pJR7

Based on the similarities between pWVO2 and several other previously described lactococcal plasmids (Hayes *et al.*, 1991; Jahns *et al.*, 1991; Horng *et al.*, 1991; Kiewiet *et al.*, 1993) we speculated that most lactococcal plasmids, other than those of the RC-type, might contain highly related, functional theta-type replication regions (Kiewiet *et al.*, 1993). Support for this idea was obtained from

Table 2. Oligonucleotides used for sequencing of replication genes from pWVO4, pWVO5 and pIL7

Nucleotides in parentheses indicate different possibilities at that location.

Oligo	Sequence	Readable sequence obtained in the dideoxy chain-termination reaction		
		pWVO4	pWVO5	pIL7
P1	AA(TA) CAA AAG CAG GTG C	−	−	+
P2	ATG CAA (GA)A(GA) CAA GC(CT) TTT T	−	+	+
P3	GAA (TC)TA (TC)AA CCA ATA CG	−	−	+
P4	(CT)AT TGT CTT TCA TAT	+	+	+
P5	GG(GT) GTC AAA GAC CAC TTG TC	+	+	+
P6	GAC AAG TGG TCT TTG AC(AC) CC	+	+	+
P7	(GA)AT ATG AAA GAC AAT (GA)	+	+	+
P8	CGT ATT GGT T(AG)T A(AG)T TC	−	+	+
P9	AAA A(GA)G CTT G(CT)T (CT)TT GCA T	+	+	+
P10	CAA GGT (TCA) (TA) (GA) CAC CTG CTT TT	+	+	+

Southern hybridizations in which the *repB* gene of pWVO2 was used as a probe against the entire plasmid content of *L. lactis* subsp. *cremoris* Wg2. This resulted in cross-hybridizations with pWVO2 as well as pWVO3, pWVO4 and pWVO5 (results not shown). To study whether pJRO4 and pJRO5, which were obtained from pWVO4 and pWVO5, as well as pJR7, contained replication regions similar to that of pWVO2, a set of ten oligonucleotide primers (Table 2) was designed on the basis of conserved regions in the nucleotide sequences of the *rep* genes of pCI305, pSK11L, pSL2 and pWVO2. With most of the oligonucleotides, clear sequence ladders were obtained (Table 2). To complete the sequence for both strands, additional oligonucleotides, based on the sequences obtained, were used. Analysis of the resulting sequences in each case revealed an ORF, similar to that of the *rep* genes of the plasmids from which the oligonucleotides were designed. Insertion of random λ DNA fragments into the *Sna*BI site in this ORF of pJRO4, or the *Hin*dIII site in the corresponding ORF of pJRO5, abolished replication in *L. lactis*. The minimal region required for replication of pJR7 was determined by creating deletions from either side of the cloned fragment with Exo III and mung bean nuclease and subsequent transformation to *L. lactis*. Deletions in one direction were created using the *Sac*I site as the 3′ protected end and the *Eco*47-3 site as the 5′ starting point for Exo III. Deletions in the other direction were created with the *Dra*III site as the 3′ protected end and the *Mlu*I site as the 5′ starting point. The deletion end-points and the entire upstream region of the pIL7-derived ORF of pJR7 were sequenced using the M13 universal and reverse primers (Short *et al.*, 1988). Replication of pJR7 in *L. lactis* was abolished when deletions removed the last 153 nucleotides from this ORF. These results show that the respective ORF in each of the analysed replicons is required for replication.

By analogy with similar regions of pCI305, pSK11L, pSL2 and pWVO2, the described ORFs of pJRO4, pJRO5 and pJR7 were denoted *repB*. In Fig. 1 the deduced amino acid sequences of the RepB proteins of pWVO2, pWVO4, pWVO5 (from strain Wg2) and pIL7 (from strain IL594) are compared with those of the equivalent proteins of other replicons. The sequences show an overall identity of approximately 30%. Conservation was extremely high in the N-terminal and central regions. The levels of identity are even higher for plasmids of certain subgroups. For instance, the levels of identity amount to 60–80% for the RepB proteins of pWVO2, pWVO4 and pWVO5, which are derived from the same strain.

Additional sequences of the region upstream of *repB* of pWVO4 and pWVO5 were determined by primer walking using oligonucleotides based on the sequences obtained. The sequence of this region of pJR7 could be determined with universal and reverse primer by using deletion derivatives, obtained from the Exo III and mung bean nuclease reactions. The corresponding regions of pWVO2 (Kiewiet *et al.*, 1993) and pCI305 (Hayes *et al.*, 1991) have previously been shown to be required for replication. Sequences similar to those determined for the previously mentioned lactococcal plasmids were found (Fig. 2). Both contain a 22 bp sequence tandemly repeated three-and-a-half times. Comparison of all repeats revealed a consensus sequence, reading 5′ ANN(G/C)(T/C)N(T/A)NAAAA-AAC(T/G)(G/A)TGTAT-3′. In addition, two regions of dyad symmetry, overlapping the putative promoter region, were identified. The inverted repeat downstream of the −10 region is highly conserved in all three pWVO2-related plasmids of *L. lactis* subsp. *cremoris* Wg2, as well as in most other lactococcal plasmids described. The region upstream of the 22 bp repeats carries a number of AT-rich stretches separated by GC-clusters. The

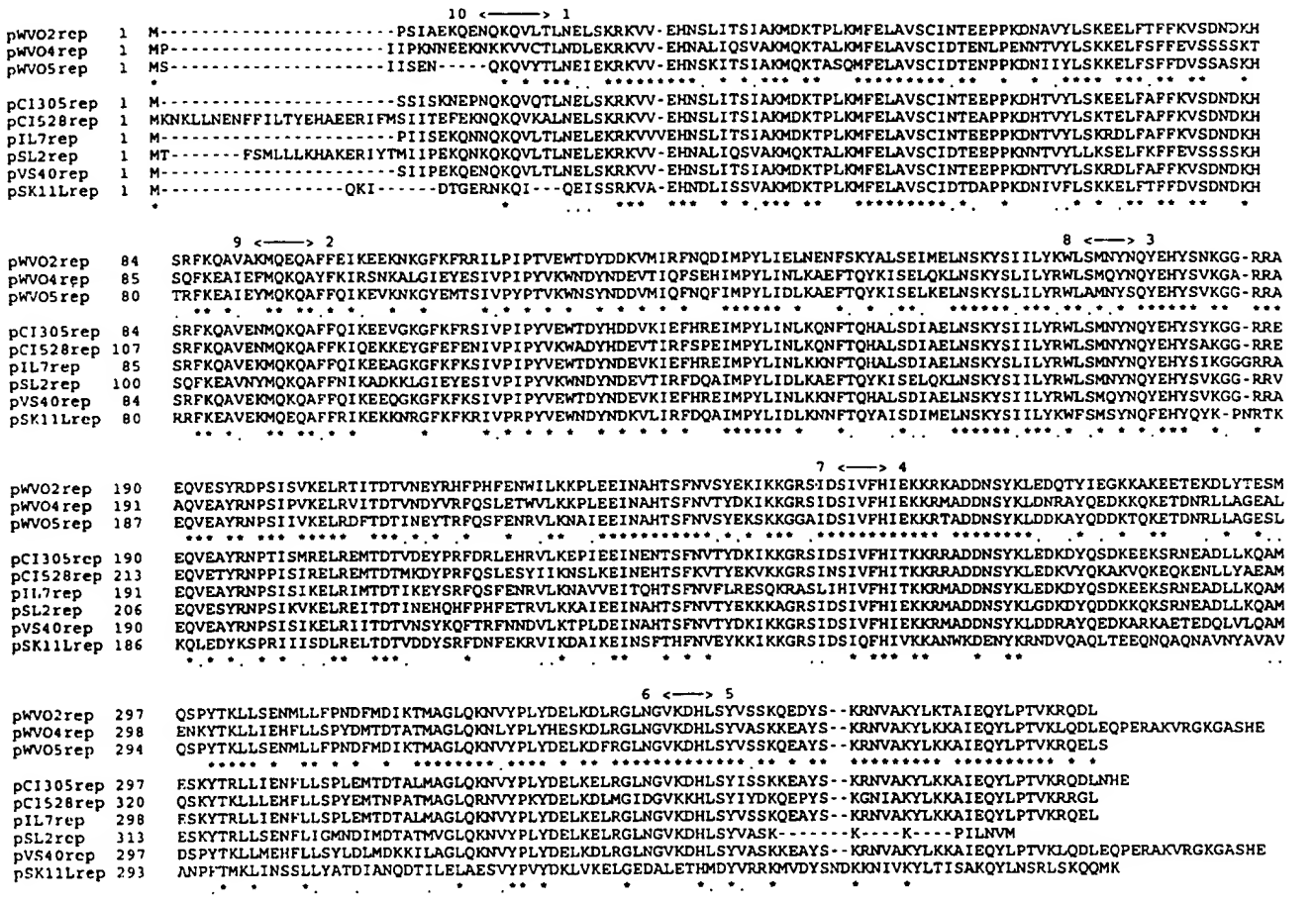

Fig. 1. Alignment of deduced amino acid sequences of RepB proteins specified by lactococcal plasmids pWVO2 (Kiewiet *et al.*, 1993), pWVO4, pWVO5, pCI305 (Hayes *et al.*, 1991), pCI528 (GenBank accession number L06274), pIL7, pSL2 (Horng *et al.*, 1991), pVS40 (GenBank accession number L02920) and pSK11L (Jahns *et al.*, 1991). The one-letter code is used. Numbers to the left of the sequences indicate the position of the first (N-terminal) aa residue of each protein. The first three plasmids (pWVO2, pWVO4 and pWVO5) were derived from the same strain (*L. lactis* subsp. *cremoris* Wg2). Identical amino acids in all sequences are indicated with an asterisk, similar amino acids with a dot. The relative positions of the primers listed in Table 2 are indicated above the sequence. Directions for primer extension are indicated by arrowheads.

nucleotide sequences of these stretches show a very high level of conservation (Fig. 2). As for pWVO2 and pCI305, this region was determined to be part of the minimal region required for replication of pJR7. Replication of derivatives obtained from the Exo III and mung bean nuclease reaction was abolished when deletions extended within this region. The smallest derivative that still supported replication extended 134 bp upstream of the region as represented in Fig. 2.

Presence of an additional ORF downstream of *repB*

Immediately downstream of the *repB* gene, the start of another ORF could be detected in the sequences of pJRO4, pJRO5 and pJR7. The entire sequence of this ORF was determined for pJR7 and found to encode a putative protein of 208 amino acids (Fig. 3a). The putative start codon (ATG) of this ORF, denoted ORF X, overlaps with the stop codon (TGA) of the upstream *repB* gene, which is indicative of translational coupling between these genes. A similar start can be identified in the related ORF X sequences of most other lactococcal plasmids (Fig. 3b). In the sequence of pWVO2 only the first 14 codons of ORF X are present. The start of ORF X in all the plasmids analysed is preceded by an appropriate Shine–Dalgarno sequence (Van de Guchte *et al.*, 1992). All deduced amino acid sequences were highly similar and showed an α-helix–turn–α-helix motif between amino acid residues 6 and 28, indicating a DNA-binding potential for this region. Deletion of this ORF from pJR7 by the Exo III and mung bean nuclease method did not affect replication. No putative transcriptional terminator could be identified downstream of either *repB* or ORF X. The observation that ORF X can be deleted from pJR7 without affecting replication, and the fact that this ORF is almost completely absent from pWVO2, suggest that it is not essential for replication.

repB of pWVO2 can complement *repB* of pWVO5

A 3·9 kb *Hin*dIII fragment of pJRO5, contained entirely within the 7 kb *Eco*RI fragment of pWVO5, which carried

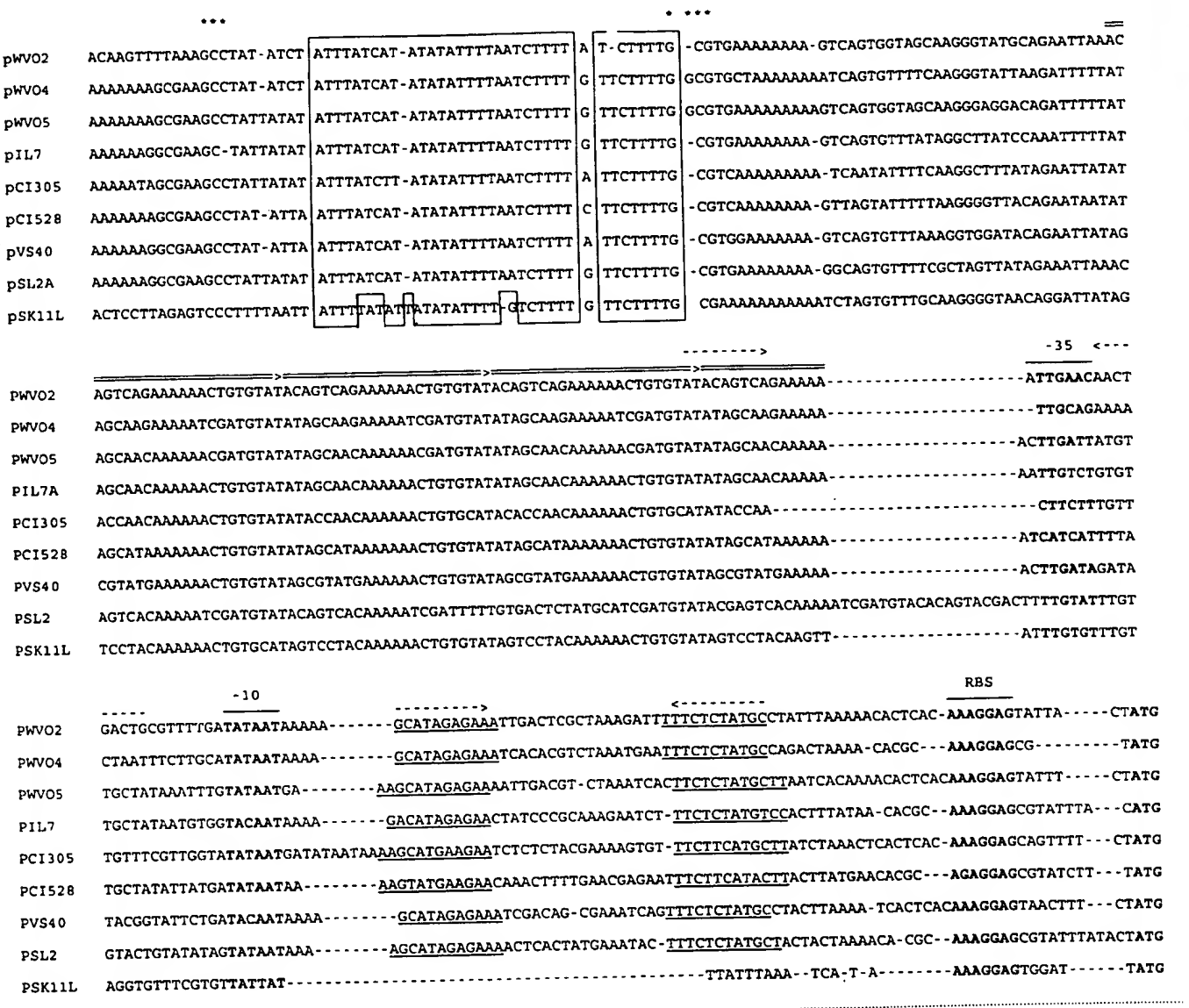

Fig. 2. Alignment of the region upstream of *repB* of the plasmids described in Fig. 1. Regions with strong sequence conservation are boxed. GC-regions, flanking AT-rich regions, are indicated with asterisks. The 22 bp sequences repeated three-and-a-half times are indicated by double arrows. Inverted repeat regions are indicated by dashed arrows. Putative transcription and translation signals are indicated in bold and by single lines.

the region upstream of the *repB* gene and the first 85 codons of the *repB* gene, was cloned into the *Hin*dIII site of pMTL23E and propagated in *E. coli*. This upstream region is essential for replication of pCI305 (Hayes *et al.*, 1991) and pWVO2 (Kiewiet *et al.*, 1993) as well as pJR7. The resulting plasmid, denoted pJRO55, was no longer capable of autonomous replication in *L. lactis*. pJRO55 was then used to transform an *L. lactis* strain carrying only pWVO2. As a control, pJRO5 was used to transform pWVO2-containing *L. lactis* cells. Transformants were selected on M17 agar supplemented with erythromycin. The transformation efficiency with pJRO55 was about 10-fold lower than with pJRO5. Several erythromycin-resistant transformants were analysed for their plasmid content. pJRO5 could clearly be detected on agarose gels in small-scale plasmid isolations (1·5 ml culture) of *L. lactis*, while pJRO55 could only be detected using large-scale plasmid isolations (100 ml culture). On the basis of ethidium bromide fluorescence intensities on agarose gels we estimated the copy number of pJRO55 in these cells to be less than one per chromosome equivalent. To verify that plasmid pJRO55 was intact and that Em^R transformants were not the result of recombination between pWVO2 and pJRO55, plasmid DNA from several erythromycin-resistant transformants was isolated and used to transform *E. coli*. Ten of the transformants were analysed for their plasmid content. All appeared to contain intact pJRO55. These results indicate that the *repB* gene of pWVO2, when supplied *in trans*, can support the replication of pJRO55, which lacks a functional copy of its own *repB* gene. In addition, this experiment provides further proof that the upstream region of *repB* is required

(a) ORF X pIL7

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ATGATGAGTGAAAAGTTAAAGACAATGAAAGAGCTTGCAGATGAGTTGAGTGTTACAAAAGAAAATATTCAATATCACTACCAAAGGTTACCAA
 M  M  S  E  K  L  K  T  M  K  E  L  A  D  E  L  S  V  T  K  E  N  I  Q  Y  H  Y  Q  R  L  P
start
AAGAATTACAGATTAAAACCCGTTCCAATGGGTCTAATCTAATAAATTCTAAAGCAGAAAAAATAATTTTAGGTAAAGTAGAAAGTGGTAGCAA
K  E  L  Q  I  K  T  R  S  N  G  S  N  L  I  N  S  K  A  E  K  I  I  L  G  K  V  E  S  G  S  K

ATCAAATACAAAAGACCAACAAATAAGTAGCAAAGAAAATTTTGAAGATGAAAAATTAGATAATCCCTTTATTAATAGATTAATCAAAGAAGTT
  S  N  T  K  D  Q  Q  I  S  S  K  E  N  F  E  D  E  K  L  D  N  P  F  I  N  R  L  I  K  E  V

GAGTATTTGAAAATAGACAAGGATAAAATCATATCTACCAAAGACCAACAAATAAGTAGCAAAGACCAACAAATAGAAAAACTAACTAATTTGT
 E  Y  L  K  I  D  K  D  K  I  I  S  T  K  D  Q  Q  I  S  S  K  D  Q  Q  I  E  K  L  T  N  L

TAGACCAGCAACAACGTTTAGCCTTGCAGGATAAAAAGTTACTTGATGAGTATAAGTCAGAAATCAATGACTTAAAAGCTCTTAAAATGCCTAC
L  D  Q  Q  Q  R  L  A  L  Q  D  K  K  L  L  D  E  Y  K  S  E  I  N  D  L  K  A  L  K  M  P  T

ACAGGAAACAGAATTCAAACACTTAGACAATCAATATAAAGATGAAGTGAACGCTCTTAAAGAGAAGTTGGAAAATTTTCAGGAACAAATCAAA
  Q  E  T  E  F  K  H  L  D  N  Q  Y  K  D  E  V  N  A  L  K  E  K  L  E  N  F  Q  E  Q  I  K

GTTCAAAAAAGGATAGAAGAACAAGAAAAACCAAGAAAGTGGTGGGGACTATGGCGAAAATAG
 V  Q  K  R  I  E  E  Q  E  K  P  R  K  W  W  G  L  W  R  K end
```

(b)

```
pWV05        ------
CTACCTACGGTTAAAAGGCAGGAATTATCATGAGTGAGAATTTAAAAACTATTAAGGAGCTT
 L  P  T  V  K  R  Q  E  L  S ——
                            M  S  E  N  L  K  T  I  K  E  L

pWV04           -----
TGCAAAGGTTCGAGGTAAAGGTGCAAGCCATGAGTGAGAATTTAAAAACGATTAAGGAGCTT
  A  K  V  R  G  K  G  A  S  H  E ——
                            M  S  E  N  L  K  T  I  K  E  L

pWV02           -----
TACCTACCAACGGTTAAAAGGCAGGACTTATGATTAGTGAAAAGTTAAAGACAATCAAAGAG
 Y  L  P  T  V  K  R  Q  D  L ——
                            M  I  S  E  K  L  K  T  I  K  E

pIL7            -----
TATCTACCTACGGTTAAAAGGCAGGAATTATGATGAGTGAAAAGTTAAAGACAATGAAAGAG
 Y  L  P  T  V  K  R  Q  E  L ——
                            M  M  S  E  K  L  K  T  M  K  E

pCI305     -----
ACCTACGGTTAAAAGGCAGGACTTAAACCATGAGTGAGAACTTAAAAAACGATTAAAGAGTG
  P  T  V  K  R  Q  D  L  N  H  E ——
                            M  S  E  N  L  K  T  I  K  E  L

pCI528        -----
CTACCAACGGTTAAAAGGCGGGGATTATAATGAGTGATAATTTAAAAACCATAAAGGAGCTA
  Y  Q  R  L  K  G  G  D  Y  N  E ——
                            M  S  D  N  L  K  T  I  K  E  L

pVS40           -----
TGCAAAGGTTCGAGGTAAAGGTGCAAGCCATGAGTGAGAATTTAAAAACGATTAAGGAGCTT
  A  K  V  R  G  K  G  A  S  H  E ——
                            M  S  E  N  L  K  T  I  K  E  L

pSL2       -----
GCCAACGGTTAAAAGGCAGGACTTAAACCATGAGTGAAGACTTAAAAACGATAAAAGAGTTG
  P  T  V  K  R  Q  D  L  N  H  E ——
                            M  S  E  D  L  K  T  I  K  E  L
```

Fig. 3. (a) Complete nucleotide sequence of ORF X of pIL7. The deduced amino acid sequence is presented below the sequence in the single-letter code. (b) Probable translational coupling between the *repB* genes of eight plasmids and ORF X. The putative Shine–Dalgarno sequence is indicated by a dashed line above the sequence. The stop codon of the *rep* gene is indicated with a solid line below the sequence. ORF X of pWV02 ends after the 14th codon. From the nucleotide sequence of pSL2, as obtained from GenBank (accession number X56550), the ORF encoding *repB* ends at the position indicated in Fig. 1. Deletion of a single base in the 3′ region of the nucleotide sequence of this gene, however, would extend the ORF to the location indicated here. In that case, ORF X would also be translationally coupled to the *repB* gene.

for replication. In the absence of selective pressure pJRO55 was rapidly lost from the culture, whereas pJRO5 was stably maintained. Loss of pWV02 was never observed.

Southern hybridizations of plasmids from various lactococcal strains

Hayes *et al.* (1991) showed that the type of replicon described here is widespread among lactococci. We tested an additional set of ten *L. lactis* strains for the presence of plasmids with a similar replicon. A 1 kb PCR fragment of the replication gene *repB* of pWV02, obtained with primers 1 and 6 (Table 2), was used as a probe. At least one plasmid in each strain tested was found to hybridize to this fragment (Fig. 4).

DISCUSSION

In the present work we have studied relationships between lactococcal plasmids which are not of the RC type. The most significant conclusions are: (1) that many of these plasmids contain a functional replication region that is part of a family of highly related replicons; and (2) that different plasmids containing these replication regions co-exist in one and the same *Lactococcus* strain. We showed that at least three out of five different plasmids present in *L. lactis* subsp. *cremoris* Wg2 contain a functional replication region of this family. One of these plasmids

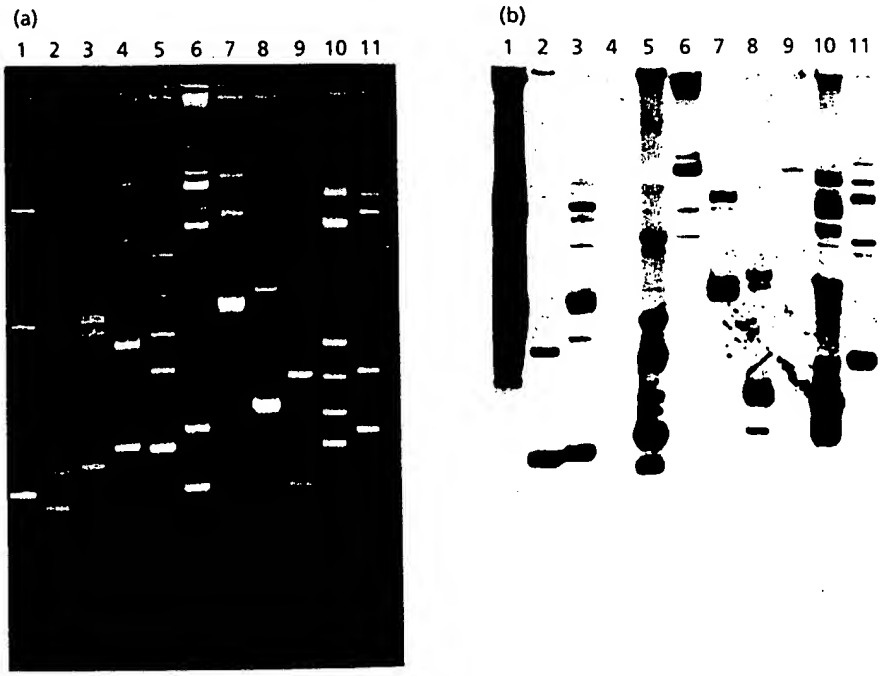

Fig. 4. Distribution of the *repB*-like replication gene in a number of lactococcal strains. (a) Plasmid profiles of a number of natural *Lactococcus* strains: 1, *L. lactis* subsp. *cremoris* Wg2; 2, *L. lactis* subsp. *cremoris* H61; 3, *L. lactis* subsp. *cremoris* F16; 4, *L. lactis* subsp. *cremoris* Hpl; 5, *L. lactis* subsp. *cremoris* E8L; 6, *L. lactis* subsp. *cremoris* Cla2; 7, *L. lactis* subsp. *cremoris* 109; 8, *L. lactis* subsp. *lactis* L10; 9, *L. lactis* subsp. *cremoris* 916; 10, *L. lactis* subsp. *lactis* P8-2-47; 11, *L. lactis* subsp. *lactis* 9B4. (b) Fluorograph of Southern hybridization of the DNA in (a) with the 1 kb PCR fragment obtained with primers 1 and 6 of the *repB* gene of pWVO2.

(pWVO2; Kiewiet *et al.*, 1993) has been shown to replicate via a theta mechanism. This suggests that the other members of this plasmid family can replicate via a similar mechanism. The results obtained do not rule out the possibility that additional functional replication regions might exist on pWVO4, pWVO5 or pIL7. We consider this possibility unlikely, however, since with none of the restriction digests used here (including a partial *Sau*3A digest of pIL7) were alternative replicons observed.

The replicons studied in this work, and other plasmids of this family, can be divided in two segments. One segment contains the *repB* gene, encoding the RepB protein, which is essential for replication of pCI305 (Hayes *et al.*, 1991) and pWVO2 (Kiewiet *et al.*, 1993). The three plasmids analysed in the present studies also require an intact *repB* gene for replication. As shown for pCI305 and pWVO2, the other essential segment is the non-coding region (approximately 300 bp) upstream of *repB*. By analogy with Gram-negative theta-replicating plasmids, like RSF1010 (Frey *et al.*, 1992) and RK2 (Perri *et al.*, 1991), for which it has been established that a number of direct repeats are required for binding of the Rep protein, it is conceivable that the 22 bp sequence repeated three-and-a-half times also constitutes the binding region for the RepB protein in theta-replicating lactococcal plasmids and that this binding is required for initiation of replication. In this context, the observation that pWVO2 can complement a RepB deficiency of pJRO5 is of interest. This can be explained by assuming that RepB of pWVO2 can recognize the binding region of pJRO55 and initiate replication. Since RepB is also required for replication of pWVO2 and probably has a higher affinity for its cognate binding sequence, pJRO55 will be maintained at a much lower level and rapidly be lost in the absence of selective pressure. Differences in the sequence of the 22 bp repeats of plasmids of this type are likely to determine the difference in affinity of RepB. This may explain why pWVO2 and pJRO5, which carry related *repB* genes and related 22 bp repeats, can co-exist within one host strain. Affinity studies, currently in progress, between the replication proteins from this family of replicons with the different repeated sequences should give more insight into the phenomenon of plasmid (in)compatibility within one strain.

The region upstream of the 22 bp repeats is highly conserved. Deletion analysis of pWVO2 has shown that this region is required for replication (Kiewiet *et al.*, 1993). On the basis of its high AT content, this region could be the site of strand separation from which replication starts. This does not, however, require strong conservation of the nucleotide sequence. Possibly, this region is the recognition site for host-encoded functions involved in replication, such as DnaB- and DnaC-like proteins, which are known to be involved in the replication initiation of a number of theta-replicating *E. coli* plasmids (Bramhill & Kornberg, 1988).

The function of ORF X, the expression of which is probably translationally coupled to that of *repB*, is as yet

unclear. In pWVO2, the putative ORF X product will be truncated since a stop codon is present after codon 14, which is followed by a transcriptional terminator within 100 bp (Kiewiet *et al.*, 1993). No sequence errors are likely to be present in this region. The likely translational coupling of ORF X to *repB* and the deduced DNA-binding properties of the ORF X product suggest an involvement in replication. Since, however, ORF X is not present intact in pWVO2, and can be deleted from pJR7 without preventing replication, its presence is not essential.

Only a limited number of lactococcal plasmids did not hybridize to the pWVO2 *repB* probe. A number of smaller plasmids hybridized with a probe derived from pWVO1 (results not shown). The latter RC replicon is also widespread among lactococci, but the strains tested so far by us and others (Dr A. Geis, personal communication) never contained more than one member of this family of plasmids. Although we have observed that a few plasmids do not hybridize to either probe (results not shown), and these might constitute yet another family of plasmids, the present results indicate that only two families of plasmid replicons are common in lactococci: the first family contains members of the pWVO2-type theta replicons, and the second, members of the pWVO1-type RC replicons.

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